Appendix D

Microbial and Biogeochemical Process Analyses

D.1 Introduction

As part of the multiple PRB project, monitoring of the groundwater was performed to characterize various microbial and biochemical parameters. Selected samples were analyzed for a series of parameters during the first and third monthly sampling events (late May and August 2003). The parameters measured included enumeration studies using Most Probable Number (MPN) analyses for nitrate-reducers and perchlorate-reducers. Chemical analyses were conducted for nitrate, sulfate, chloride, perchlorate, chlorate and chlorite by ion chromatography (for comparison with other analyses); organic volatile fatty acid (VFA) analyses for acetate, propionate, lactate, and butyrate; ferric and ferrous iron; and specific 16S rDNA probe analysis to identify species of perchlorate reducers present. A newly developed ELISA (enzyme-linked immunosorbent assay) method is being developed to analyze for chlorite dismutase, the enzyme that degrades chlorite to produce chloride during the microbial reduction pathway of perchlorate. Results of this analysis will be available upon completion of the assay development process, and only for the August 2003 samples. The presence of this enzyme indicates that active perchlorate reduction is occurring. This appendix presents only the May results, as the August sample analysis is incomplete at this point due to the length of time required for a valid MPN assay result. An updated version of the appendix will be submitted when the data for the August samples and the ELISA analyses are available.

Both nitrate and perchlorate are reduced by specific microbial species in the environment, when sufficient electron donor (carbon) is present, but only under anaerobic conditions in a narrow redox potential range. Several researchers have shown that common soil bacteria reductively dechlorinate perchlorate, and examples of these perchlorate-reducers have been isolated (1-6). There is evidence that the mechanism may involve the same or a similar pathway to that which is responsible for microbial denitrification (7), however, until recently, very little was known about the diversity or ubiquity of microorganisms that can grow by dissimilatory chlorate or perchlorate respiration (8). Significant progress has been made in the past several years, however, and the microbial processes, as well as identification of the species responsible for them, are contributing to an expanding knowledge base. In contrast, nitrate reduction has been under investigation for many decades and is very well understood. Many classes of bacteria are capable of denitrification, and reductive dechlorination of perchlorate is also a widespread capability in microbial populations.

Perchlorate is a stable anion that is reduced in the microbial pathway of perchlorate-reducing bacteria (ClRB) to chlorate, then chlorite, and finally is dismuted to chloride and water by the microbial enzyme chlorite dismutase. This dismutation represents a central step in the reductive pathway of perchlorate and is common to all ClRB. It is mediated by the enzyme chlorite dismutase (CD), an enzyme that is known to be highly conserved among the ClRB, regardless of their phylogenetic affiliation (9). The enzyme also was shown to be present on the outer membrane of all ClRB, and was only expressed when the organisms were grown anaerobically in the presence of perchlorate or chlorate (9). Although microbial reduction of chlorate has been known for more than 7 decades (10), this metabolism was previously associated with nitrate-respiring organisms, and chlorate was considered to be a

non-specific substrate for the nitrate reductase enzyme (11, 12, 13). It is now commonly accepted that specialized microorganisms have evolved that couple growth to reduction of chlorate or perchlorate, completely reducing these compounds to chloride (e.g. 14-17). The phylogenetic diversity of these microorganisms is great, with members in the alpha, beta, gamma, and epsilon subclasses of the Proteobacteria (14-19). The major reducers of perchlorate are in the beta subclass, and are members of the *Dechloromonas* or *Dechlorosoma* genera (14, 19). In groundwater, perchlorate generally is reduced only after all available nitrate has been reduced.

The geochemical cycling of nitrogen is highly dependent on microbial processes (20,21). Two primary nitrate reduction processes are termed assimilatory and dissimilatory nitrate reduction. Assimilatory nitrate reduction is a process that provides nitrogen to the microbial cell in the form of ammonia, as a source for amino acid and nucleic acid production, which allow the cells to grow and reproduce. The enzymes involved are not inhibited in the presence of oxygen, but are regulated by ammonia concentration. High levels of ammonia in the environment usually do not occur due to the rapid incorporation of ammonia into the cell, and feedback inhibition by ammonia on the assimilatory nitrate reductase enzyme activity. Dissimilatory nitrate reduction, or nitrate respiration, occurs in the absence of oxygen. Nitrate acts in place of oxygen as a terminal electron acceptor. The presence of nitrate in contaminated water along with oxidized metals such as iron or uranium [as uranyl, U(VI)] has been shown to inhibit reduction of these metals until the nitrate has been reduced completely (22). This is because nitrate is second only to oxygen in the amount of energy that is derived by the microbial cell when nitrate reduction is coupled to organic matter oxidation, and this reduction occurs at a higher redox potential than the reduction of perchlorate, metals or radionuclides.

A wide variety of soil microbial genera are capable of dissimilatory denitrification (22-27), many from the *Proteobacteria* class. The microorganisms involved in the reduction of nitrate are well known to be facultative anaerobes that denitrify under the appropriate anoxic conditions when the preferred electron acceptor (oxygen) is exhausted. There are two types of dissimilatory nitrate reduction, only one is termed "true" denitrification. The first process is carried out by many different species of facultatively anaerobic bacteria found in soil and sediments, where nitrate is reduced to nitrite only. The resulting nitrite is either excreted, or is reduced via hydroxylamine to ammonia (nitrate ammonification) under appropriate conditions. However, it is an environmentally less significant process for the reductive removal of nitrate; its importance appears to be limited by the number of reducing equivalents that must be consumed in the system. True denitrification, the second process, is carried out in soil primarily by *Pseudomonas* and *Alcaligenes* species, although many other genera (e.g. *Rhizobium*, *Azospirillium*) are known to denitrify under certain conditions. Nitrate is sequentially converted to nitrite, nitric oxide, nitrous oxide and nitrogen gas in this pathway, where a different specific enzyme catalyzes each step in the chain, i.e. nitrate reductases, nitrite reductases, nitric oxide reductases, and nitrous oxide reductases. A recent literature review (23) demonstrated that the process is not as simple as might be expected. For instance, there is a group of microorganisms that use nitrous oxide in a respiratory process as a terminal electron acceptor. These microorganisms do not appear to have the other enzymes of the denitrification pathway (i.e. nitrate reductase, nitrite reductase, etc.) present in their cells, only the nitrous oxide reductase is present. The end products of denitrification

are usually a mixture of nitrous oxide and nitrogen gases, which are lost to the atmosphere, resulting in depletion of combined nitrogen in the soil or sediment environment.

D.2 Methods of Analysis

Enumeration Studies. The perchlorate- and nitrate-reducing populations in the samples were enumerated by most probable number (MPN) analysis, with acetate as the electron donor, using a three-tube method. Anaerobic basal medium was prepared, under a headspace of N_2-CO_2 (80-20, vol/vol.), using standard anaerobic techniques, and amended with ammonium perchlorate (5 mM) or nitrate (5 mM) as the electron acceptor, respectively, and acetate (10 mM) as the electron donor. Positives in the MPN series were identified by visual observation of optical density increase. Population numbers in cells/ml were estimated by scoring the MPN results.

Chemical Analyses. Samples were analyzed for the presence of nitrate, nitrite, sulfate, chloride, fluoride, perchlorate, chlorate, and chlorite by ion chromatography (IC). The concentration of perchlorate in samples was determined by IC coupled to suppressed conductivity using a Dionex IonPac AS 11 4x250mm column (Dionex Corporation, Sunnyvale, CA) with a 100 mM NaOH mobile phase at a flow rate of 1mL min^{-1}. The eluting perchlorate was detected by a conductivity detector (Shimadzu model: CDD - 6A) suppressed with a Dionex ASRS-Ultra operating in external water mode set at 300 mA. The method detection limit (MDL) for perchlorate is 4 ppb. Chlorate, chlorite, chloride, sulfate, nitrate, nitrite and fluoride in the water were determined using a Dionex DX500 ion chromatograph (Dionex Corporation, Sunnyvale, CA) equipped with a GP50 gradient pump, CD20 conductivity detector, ASRS-Ultra for suppressed conductivity, and PeakNet 6 controlling software. An IonPac AS9-SC 4x250 mm column was used for analysis with bicarbonate buffer containing 2 mM sodium carbonate and 0.75 mM sodium bicarbonate as the eluent at a flow rate of 2 mL min^{-1}. The SRS current was set at 100 mA for all of the analyses. Detection limits were: chloride, 20 ppb; chlorate, 10 ppb; chlorite, 100 ppb, nitrate, 20 ppb; nitrite, 20 ppb; and sulfate, 20 ppb. Most of these analytes were also determined by the project as part of the main testing suite. These results are confirmatory only and were not used in the analysis of PRB performance presented in the main body of the report and Appendix C. There is reasonable agreement with these results and analytical results for anions presented in Section 5.2 and Appendix C.

Organic Volatile Fatty Acid (VFA) Analyses. Acetate, propionate, lactate, and butyrate concentrations were determined by HPLC (Shimadzu, Model: SPD-10A), equipped with UV-VIS detector, at a wavelength of 210 nm using a HL-75H^+ cation-exchange column (Hamilton, Model #79476), using a mobile phase of 0.016 N H_2SO_4 at a flow rate of 0.4 mL min^{-1}. Samples were prepared for analysis by prior filtration through 0.2 μm sterile filters (nylon, Fisher No. 09-719C) to remove particulates. Results were compared against concentration standards prepared at the time of analysis.

Ferrous (Fe^{2+}) and Ferric (Fe^{3+}) Iron Analyses. The Fe(II) and Fe(III) content of samples was analyzed by standard colorimetric assay with ferrozine after extraction in 0.5 M HCl. The

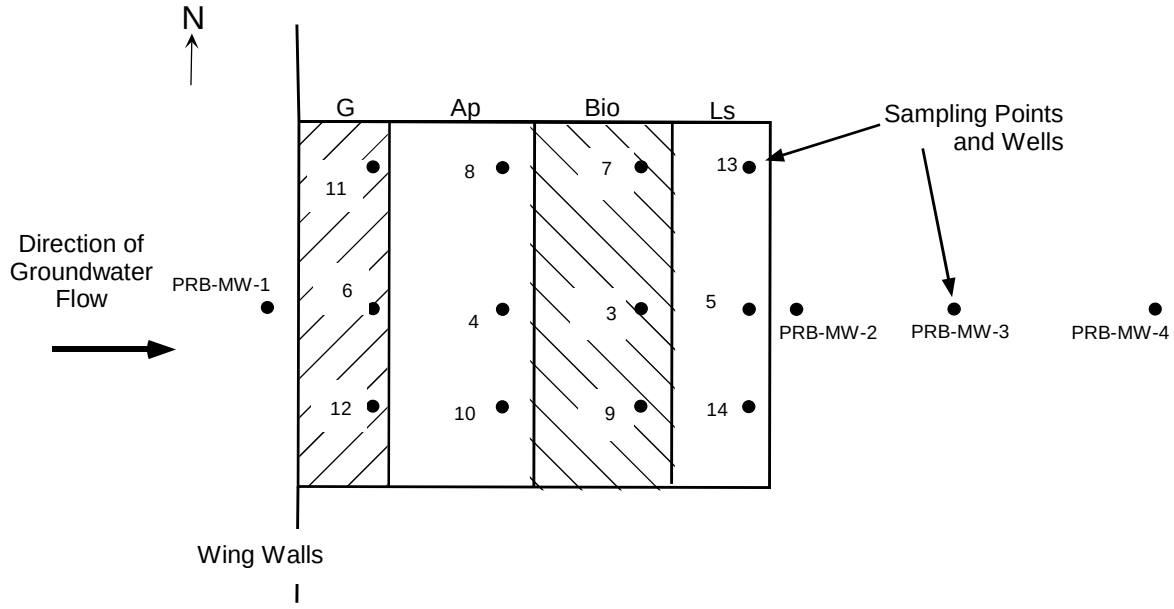
absorbance at 562 nm was measured following the method of Lovely and Phillips,1987 (28). Concentration standards were used to calibrate the method.

Identification of Dominant Perchlorate Reducers. The predominant perchlorate-reducing bacterial species in the groundwater were identified. The method (29) involved extraction of the genomic DNA from the highest dilution tubes of the MPN series that showed growth. Both perchlorate and nitrate MPN tubes were extracted in order to determine if any species were present that reduced both contaminants. The 16S rDNA was amplified by polymerase chain reaction (PCR) using 16S rDNA probes specific to the *Dechloromonas, Dechlorosoma,* and *Dechlorospirillum* groups (*Dechloromonas* CKB-type; *Dechloromonas* RCB-type; *Dechlorosoma* PS-type, and *Dechlorospirillum* WD-type), which are known to be the dominant perchlorate reducers in most environments. Positives were identified by the presence of PCR amplification products in agarose gels by electrophoresis. Control analyses were performed by amplification of eubacterial 16S rDNA in the samples with universal primer sets (8F: 5'- AGAGTTTGATCCTGGCTCAG-3'; 1525R: 5'-AAGGAGGTGATCCAGCC-3') to ensure successful extraction of DNA from the samples.

Chlorite Dismutase ELISA Analysis. Active perchlorate degraders are detected using an ELISA method to detect the chlorite dismutase enzyme. This enzyme catalyzes the reaction whereby chlorite is converted to chloride, and is highly specific to perchlorate reducing microorganisms. The method uses an antibody (rabbit) specific for the enzyme, coupled to a detector enzyme (peroxidase). More information will be supplied upon publication of the method, and results will be provided in an addendum to the report.

D.3 Results

Analytical results are presented in Tables D-1 through D-6 for each of the above analytical methods. Samples were analyzed from both late May and August 2003 sampling events, however, only the May results are currently available. Groundwater samples were collected from the following wells and ports install within, upgradient, and downgradient of the multiple PRB (refer to location map on next page):
PRB – 1: Downgradient well, MCO-5,
PRB – 2: Upgradient well, MCO-4B,
PRB – 3: Center well in bio-barrier cell,
PRB – 4: Center well in phosphate rock (apatite) cell,
PRB – 6: Center well in gravel cell, and
PRB – 7: Perimeter well in bio-barrier (north of center).



Schematic diagram depicting PRB water sampling ports and monitor wells. Analytical tables and discussion below are keyed to this diagram. G = gravel (scoria) cell, Ap – phosphate rock cell, Bio = biobarrier, Ls = limestone cell. Each layer contains 3 water sample ports.

Table D-1. Estimates of Populations of Nitrate- and Perchlorate-Reducing Bacteria Present in Groundwater Obtained from the Multiple PRB During May 2003 (results calculated from MPN analysis)

Site Name	MPN (NO_3^-) (Cells/mL)-May 03	MPN (ClO_4^-) (Cells/mL)-May 03
PRB(1)-1	$4.27 \pm 2.14 \times 10^6$	ND[a]
PRB(1)-2	$2.39 \pm 1.74 \times 10^6$	$4.27 \pm 2.14 \times 10^1$
PRB(1)-3	$7.49 \pm 3.35 \times 10^6$	$2.31 \pm 1.33 \times 10^1$
PRB(1)-4	$1.49 \pm 0.61 \times 10^6$	$0.92 \pm 0.65 \times 10^1$
PRB(1)-6	$2.39 \pm 1.74 \times 10^6$	ND
PRB(1)-7	$2.39 \pm 1.74 \times 10^6$	$2.31 \pm 1.33 \times 10^1$

[a] ND = None detected

Table D-2. Ion Chromatographic Analysis of Anions, and pH in Groundwater Obtained from the Multiple PRB in May 2003

Site Name	pH	ClO_4^- (ppm)	SO_4^{2-} (ppm)	NO_3^- (ppm)	Cl^- (ppm)	F^- (ppm)
RB(1)-1	6.96	Not analyzed	49.32	11.27	40.04	1.09
PRB(1)-2	7.15	0.0198	53.86	12.27	44.84	1.61
PRB(1)-3	6.56	ND	9.87	0.24	35.29	1.61
PRB(1)-4	6.06	ND	ND	0.03	37.01	ND
PRB(1)-6	6.90	Not analyzed	53.05	10.87	46.86	1.26
PRB(1)-7	6.85	ND	43.42	0.17	44.18	1.06

Table D-3. Organic Volatile Fatty Acids Present in Groundwater Samples from the Multiple PRB in May 2003

Site Name	Acetate (mM)	Propionate (mM)	Butyrate (mM)
PRB (1)-1	ND	ND	ND
PRB (1)-2	ND	ND	ND
PRB (1)-3	1.38	0.14	ND
PRB (1)-4	3.85	1.67	ND
PRB (1)-6	ND	ND	ND
PRB (1)-7	ND	ND	ND

Table D-4. Iron Analyses for Groundwater Samples from the Multiple PRB in May 2003

Site Name	Fe^{2+} (mM)	Fe^{3+} (mM)
PRB (1)-1	ND	0.5
PRB (1)-2	0.6	ND
PRB (1)-3	0	0.9
PRB (1)-4	1.2	ND
PRB (1)-6	ND	0.7
PRB (1)-7	ND	0.5

Table D-5. Identification of Dominant Microbial Species by MPN-PCR on the Highest Dilution MPN Culture Under Perchlorate-Reducing Conditions

Site Name	Universal Primers (control)	WD Primers	CKB Primers	RCB Primers	PS Primers
PRB (1)-2	(+)	(–)	(–)	(+)	(–)
PRB (1)-3	(+)	(–)	(–)	(+)	(–)
PRB (1)-4	(+)	(–)	(–)	(+)	(–)
PRB (1)-7	(+)	(–)	(–)	(+)	(–)

(+) 16S DNA was successfully amplified.
(-) 16S DNA was not amplified.

Table D-6. Identification of Perchlorate-Reducing Species by MPN-PCR on the Highest Dilution MPN under Nitrate-Reducing Conditions

Site Name	Universal Primers (control)	WD Primers	CKB Primers	RCB Primers	PS Primers
PRB (1)-1	(+)	(–)	(–)	(–)	(–)
PRB (1)-2	(+)	(–)	(–)	(–)	(–)
PRB (1)-3	(+)	(–)	(–)	(–)	(–)
PRB (1)-4	(+)	(–)	(–)	(–)	(–)
PRB (1)-6	(+)	(–)	(–)	(–)	(–)
PRB (1)-7	(+)	(–)	(–)	(–)	(–)

(+) means 16S DNA was successfully amplified.
(-) means no 16S DNA was amplified.

D.4 Discussion

The results presented above show that there is active degradation of nitrate and perchlorate occurring in both the phosphate rock (apatite) and bio-barrier cells (samples 4 and 3,7 respectively). Total organic carbon analyses indicate a relatively high amount of carbon present in the phosphate rock (0.77%), sufficient to account for the levels of microbial activity occurring in these samples. It is expected that this organic carbon, present in the phosphate rock, will serve as a carbon source for growth, and will be rapidly consumed by indigenous bacteria. Microbial reduction in the phosphate rock cell will most likely cease in several years, but this process will continue in the bio-barrier cell.

In addition to low concentrations of nitrate and perchlorate in the groundwater samples, other evidence for active microbial reduction and growth includes both lowered pH and ORP. Decreasing ORP measurements most likely resulted from utilization of the available oxygen by microorganisms, and production of the VFAs acetate and propionate as by-products of metabolism (Table D-3). In addition, the lowered DO (see Appendix C and Section 5.2), increased ferrous iron, and decreased ferric iron (Table D-4) provide indications that reducing conditions necessary for nitrate and perchlorate reduction are present within the phosphate rock and bio-barrier cells. Evidence for iron and sulfate reduction in both the apatite and the bio-barrier is also present (Appendix C). Analyses of the north perimeter well in the bio-barrier cell (sample #7) shows similar results to those in the center well (sample #3), but

microbial activity appears to be much lower due to the smaller amount of groundwater present during this timeframe.

Results of the microbial identification studies conducted under perchlorate-reducing conditions imply that the dominant perchlorate-reducing bacteria in these samples were members of the *Dechloromonas* group and were closely related to the perchlorate-reducer *Dechloromonas aromatica* strain RCB. Results of an investigation conducted under nitrate-reducing conditions imply that nitrate reduction, in these samples, was not being mediated by members of the *Dechloromonas, Dechlorosoma,* or *Dechlorospirillum* groups (Table D-6). Enumeration results showed the presence of both nitrate- and perchlorate-reducing bacteria in all of the groundwater samples, with only a slight increase in bacterial populations in the bio-barrier cell. As time passes, it is expected that these numbers will increase, however, most bacteria that thrive in the subsurface are attached to solid surfaces. An analysis of the solid materials in the cells will be adequate to show meaningful changes in the microbial populations.

D.5 Conclusions

The multiple PRB installed in Mortandad Canyon is functioning well in that it is removing both nitrate and perchlorate to concentrations below their respective MDLs using ion chromatography. Concentrations of nitrate and perchlorate in the phosphate rock and bio-barrier cell are below their respective maximum contaminant limit (MCL) (10 ppm $N\text{-}NO_3$) and proposed groundwater risk level (4 ppb ClO_4). In addition, results of microbial characterization indicate that intermediate degradation products such as nitrite and chlorate are largely being transformed to the final products of reduction (nitrogen gas and chloride, respectively) within the groundwater residence time for the multiple PRB. A single genus of perchlorate reducer, *Dechloromonas*, was initially detected in a groundwater sample collected from the site.

Only continued monitoring for the microbial and other parameters described here will allow us to determine the lifetime of acceptable performance of the multiple PRB. Additional studies of both the solid materials and the groundwater will be necessary to thoroughly understand the physicochemical processes occurring in the PRB. These studies will also help to predict the rate of utilization and the eventual lifetime of the reactive barrier materials.

D.6 References

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